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COLD STORAGE AND CRYOPRESERVATION OF HOPS (*HUMULUS* L.) SHOOT CULTURES THROUGH APPLICATION OF STANDARD PROTOCOLS

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Summary

The USDA-ARS National Clonal Germplasm Repository (NCGR) stores the global diversity of *Humulus* for the US Plant Germplasm System as trellised plants in a field genebank. In vitro storage and cryopreservation are now considered excellent ways to provide medium and long-term storage for plant collections. Developing a new cryopreservation or cold storage protocol for every accession or genus of large multi-crop collections can be a very time consuming and long-term activity. We propose that standard cold storage and cryopreservation techniques used for other temperate crop genera would be successful for additional crops with few modifications. This study was initiated to determine if a large collection of hops germplasm could be successfully stored with techniques developed for unrelated genera. In this study we characterized the response of diverse *Humulus* genotypes to *in vitro* storage under low light at 4 °C following techniques used for strawberry and mint plants, and cryopreservation in liquid nitrogen by slow cooling with a pear protocol. The average storage time without transfer for the 70 genotypes evaluated was 14 $\pm$ 3.5 months with a range of 6 to 26 months. Mean recovery of cryopreserved shoot tips of accessions with 1-wk cold acclimation was 41% $\pm$ 18 and increased to 54% $\pm$ 13 with 2-wk cold acclimation. This demonstrates that application of a well-tested standard technique can provide a quick start for storing additional germplasm collections.

Keywords: cold acclimation, cryopreservation, germplasm, *Humulus, in vitro* storage, liquid nitrogen, slow cooling.

INTRODUCTION

The genus *Humulus* is indigenous to Europe, Asia, and North America (3) but likely originated in China, where all three species of the genus occur (19). *Humulus japonicus* Siebold & Zucc. is native to Japan, Taiwan, and China while *H. yunnanensis* Hu is native to high altitudes of the Yunan Province of China. *Humulus lupulus* L. is native to China, Europe and North America. All commercial hops were developed from *Humulus lupulus*. Most cultivars were originally derived from wild European hop selections, but many recent cultivars have North American germplasm in their pedigrees (8). Hops germplasm collections are commonly held in fields as perennial rhizomes. Diseases, insects, and

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environmental stresses put these plants at risk. In addition, virus diseases can accumulate in a field collection and be transferred to additional sites by vegetative propagation. The USDA-ARS National Clonal Germplasm Repository, Corvallis, Oregon stores nearly 700 accessions of *Humulus* germplasm collected from many sources. The accessions are held as a field collection, 62 representative (core) accessions heat treated to eliminate viruses stored in pots under screen, and as tissue cultures.

Cryopreservation is now considered a viable option for long-term (base) storage of clonally propagated germplasm (2). Developing a new cryopreservation or cold storage protocol for every accession or genus of large multi-crop collections can be a very time consuming and long-term activity (2,14). We propose that standard *in vitro* cold storage and cryopreservation techniques used for other temperate crop genera would be successful for similar crops with few modifications. This study was initiated to determine if a large collection of temperate hops germplasm could be successfully stored with techniques developed for unrelated genera. This study characterizes the response of diverse genotypes of *Humulus* germplasm to *in vitro* cold storage and to cryopreservation in liquid nitrogen following techniques used for blackberries, pears, and strawberries (4,5,6,13,14,17). Our objective was to determine the response of clonally propagated *Humulus* germplasm to standard medium- and long-term storage techniques and to store cryopreserved accessions as a base collection.

MATERIALS AND METHODS

Plant Materials: *In vitro* cultures were initiated from 0.3 to 0.5 mm meristems of heat-treated shoots from clonally propagated hops plants (1). Meristems were cultured individually in 24-well culture plates on 2 ml NCGR-HUM medium composed of Murashige and Skoog (10) salts and vitamins with 2% glucose, 4.4 μM N^6 benzyladenine, at pH 5.0 and gelled with 0.3% agar and 0.125% Gelrite. New shoots were transferred to 10 ml fresh medium in 16 x 100 mm tubes after 3 to 4 wks or 40 ml of medium in Magenta GA-7 vessels for additional growth and multiplication. Cultures were grown at 25 °C under a 16-h photoperiod (40 $\mu mol \bullet m^{-2} \bullet s^{-1}$).

Cold-Storage of In Vitro Cultures: Storage followed the technique developed for other genera but with NCGR-HUM medium (13,15,17). Plantlets (2 to 3 cm height) were transferred to two 5-chambered semi-permeable tissue-culture bags (Star-pak, Garner Enterprises, Willis, Tex) with 10 ml medium per chamber 3 wk after the last regular subculture. Storage medium was NCGR-HUM medium without growth regulators, and was gelled with 0.35% agar and 0.145% gelrite. Ten plantlets of each accession were stored, each in an individual section (15 x 150 mm) of a five-section bag. Sealed cultures were grown for 1 wk in the growth room, and then for 1 wk under the cold acclimation (CA) conditions described below. Storage was at 4 °C with a 12-h photoperiod and very low light (3 $\mu mol \bullet m^{-2} \bullet s^{-1}$). Data was taken at 4 month intervals and each bag of plantlets was rated on a 0 to 5 scale. Ratings were: 5, dark green leaves and stems, no etiolation, base green; 4, green leaves and stems, little etiolation; 3, shoot tips and upper leaves green, etiolation present, base green; 2, shoot tip green, leaves and stems mostly brown, base may be brown; 1, plantlet mostly brown, only extreme shoot tip green, much of base dark brown; 0, all of plantlet brown, no visible green on shoot tip. Plantlets were removed for repropagation when ratings reached ≤ 2 and the length of storage was noted (13,15,17). One storage period was studied for 70 accessions and the 10 plantlets of each accession were treated as a group.

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Cold Acclimation (CA): Mother plants on NCGR-HUM medium were CA for 1 wk for cold storage and 2 wk for cryopreservation in a growth chamber with temperature/photoperiod settings of -1°C 16-h dark/ 22°C 8-h light (10 $\mu mol \cdot m^{-2} \cdot s^{-1}$) as the standard treatment (4,5,6,11, 17).

Cryopreservation: Shoot tips (0.8 to 1.0 mm) of 1-wk or 2-wk CA plantlets of each genotype were dissected and precultured on NCGR-HUM medium with 0.35% agar and 0.18% Gelrite and 5% dimethyl sulfoxide (DMSO) for 48 hr under the same temperature conditions as the parent shoots. Samples were subjected to slow cooling (12). Shoot tips were transferred to 0.25 ml liquid MS medium (10) in 1.2 ml plastic cryovials on ice and the cryoprotectant PGD (7), a mixture of 10% (w/w) each of polyethylene glycol (MW 8000), glucose, and DMSO in liquid MS medium, was added drop wise up to 1.2 ml over 30 min (12). After 30 min equilibration on ice, the shoot tips were frozen to –40 °C at 0.1 °C/min in a programmable freezer (Cryomed, Forma Scientific, Mt. Clemens, Mich.) with nucleation at – 9 °C and immersed in liquid nitrogen. Vials were thawed in 45 °C water for 1 min, then in 23 °C water for 2 min. The cryoprotectant was removed and replaced with liquid MS medium. Shoot tips were plated in 24-cell plates with 2 ml NCGR-HUM medium per cell (Costar, Cambridge, Mass.) for recovery. Regrowth data were taken 6 wks after thawing. Each experiment included 20 shoot tips in one vial for each treatment and five shoot tips for unfrozen controls, with at least three replications of the experiment (n = 60). For storage, 150 shoot tips of each accession were processed. Two vials of 25 shoot tips served as cryopreserved controls (one thawed at NCGR and one thawed at the National Center for Germplasm Resources Preservation). Ten vials of 10 shoot tips were stored as a base collection.

RESULTS AND DISCUSSION

Cold storage of in vitro cultures: Plantlets remained in cold storage for an average of 14.1 $\pm$ 3.5 months. The range for individual accessions was 6 to 26 months (Table 1). There was significant difference in length of storage for cultivars (14.6 ± 3.4) vs. wild accessions (12.6 ± 3.2). The frequency graph of condition ratings for the 70 accessions shows ratings decreasing by about one point for each inventory period for the majority of plants (Fig. 1). Long-storing plants remained at the "3" rating level (shoot tips and upper leaves green, etiolation present, base green) for longer periods than the average plant. This technique was originally optimized for *Fragaria* (strawberry) (18 mo $\pm$ 6), and applied to *Rubus* (27 mo $\pm$ 14), and *Pyrus* (32 mo $\pm$ 12) (13,15,17). *Humulus* has a very different growth form and is in a separate family from the *Rosaceous* genera for which the method was developed, however the accessions remained in good condition for an average of more than one year. This large group of accessions moved through one storage cycle with good results using standard storage techniques developed for unrelated genera.

It is likely that additional improvements can be made to increase the length of storage for individual *Humulus* accessions, but for the majority the technique is directly applicable. When optimizing a medium-term *in vitro*-storage system the age, size and physiology of the plants must be considered as well as temperature of storage, cold acclimation, roots, single or multiple shoots, light intensity and quality and storage medium (2,9,13,15,16,17). These factors require long periods of study to fully optimize storage for a genus, however, the standard technique can be used quickly and with generally good results almost immediately following the decision to store a new type of plant. With proper monitoring of the stored collection at regular intervals, even the poorest performing genotypes can be safely held.

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Table 1: Length of *in vitro* cold-storage for one *Humulus japonicus* and 69 *Humulus lupulus* accessions. Plantlets were sealed in semi-permeable plastic bags, cold acclimated and held at 4 °C with 12 hr of low light during storage. Plantlets were removed from storage and regrown when ratings dropped to ≤ 2.

Local ID	Species or Cultivar	Months Stored		Local ID	Species or Cultivar	Months Stored
570.002	BC-11	12		246.003	Kirin II	15
114.002	Blue Northern Brewer	17		915.004	Kitamidori	15
132.004	Bullion 10A	15		205.002	Landhopfen	16
196.002	Cascade	21		11.003	Late Cluster seedling	12
56.002	Comet S (Comet x OP)	14		77.004	Late Cluster seedling	12
720.002	Crystal	12		721.002	Liberty	14
134.004	Eroica	12		647.002	Lubelska	14
812.003	Fuggle H	16		597.002	Mt. Hood	11
241.003	Fuggle Tetraploid	14		530.002	Perle	9
930.003	Furano Ace	14		198.002	Precoce de Bourgogne	18
211.003	Golden Star	15		148.002	Pride of Kent	19
579.003	*H. japonicus* Tug Fork # 11	14		635.002	Saazer 36	11
542.002	*H. lupulus*	11		201.002	Savinja Golding	19
60.003	*H. lupulus* Arizona 1-2 (Wild American)	20		191.002	Shinsuwase	14
489.002	*H. lupulus* Brownville, Nebraska	8		816.002	Spalter Select	18
492.001	*H. lupulus* Brownville, Nebraska	12		129.001	Styrian Golding	18
46.002	*H. lupulus* Colorado 1-1 (Wild American)	10		814.002	Sunbeam	10
8.003	*H. lupulus* Colorado 2-1 (Wild American)	12		626.002	SuperAlpha	14
106.001	*H. lupulus* Colorado 3-1 (Wild American)	18		192.003	Tardif de Bourgogne	14
112.003	*H. lupulus* Colorado 7-2 (Wild American)	14		197.002	Tettnanger	15
504.002	*H. lupulus* Millville, Iowa	13		13.004	USDA 19085M (LhS x GCI-Fu S)	26
509.001	*H. lupulus* Millville, Iowa	9		53.003	USDA 19173M (SSP x LCS)	18
755.002	*H. lupulus* Missouri 3 (Wild American)	14		19.002	USDA 21072M	15
496.002	*H. lupulus* Rulo, Nebraska	8		372.002	USDA 21121 (19005 x 19046M)	17
72.001	*H. lupulus* Utah 526-5 (Wild American)	14		365.002	USDA 21125 (19005 x 19046M)	9
58.002	*H. lupulus* Wild Yugoslavian 17/17	14		373.002	USDA 21127 (19005 x 19046M)	11
63.002	*H. lupulus* Wild Yugoslavian 3/3	14		128.003	Willamette	13
44.002	*H. lupulus* Wisconsin selection	10		920.004	Wuerttemberger	15
91.002	*H. lupulus* Wyoming 3-1 (Wild American)	12		182.002	Wye Challenger	9
819.006	Hallertauer Gold	18		144.003	Wye Saxon	18
815.002	Hallertauer Magnum	17		88.004	Wye Target	15
203.002	Hallertauer Mittelfruher	12		204.003	Yugoslavia Golding	15
820.005	Hallertauer Tradition	17		16.002	Zattler seedling	12
613.002	Hersbrucker-8	17		49.002	Zattler seedling	6
645.002	Hersbrucker-alpha	14			Mean Storage	14.11
126.003	Huller [Hueller Bitterer]	17			St. Dev.	3.45

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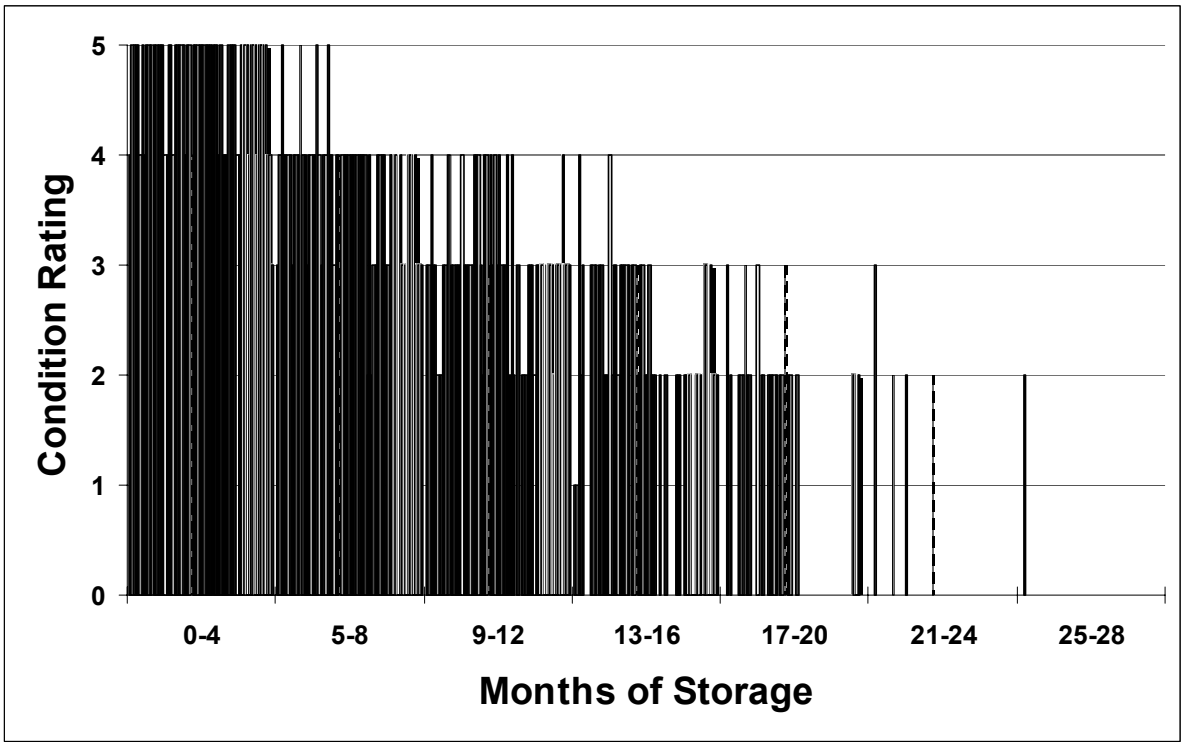
Figure 1: Frequency of growth condition ratings for 70 cold-stored *Humulus* accessions by length of storage. Growth ratings were 5 (excellent) to 0 (dead).



Cryopreservation: The slow-cooling procedure developed for *Pyrus* (5,6,12,17) was successful for all accessions tested. Initial testing of *Humulus* cultivars and wild accessions showed genotype variation, and recovery with 1-wk CA ranged from poor for 4 accessions to moderate for the other 9 (Fig. 2). The mean regrowth of *Humulus* meristems was 41 $\pm$ 18 % with the lowest recovery at 7% and a high 65%. This initial protocol was chosen because in earlier studies 1-wk CA produced twice the regrowth of many *Pyrus* and *Rubus* meristems compared to low or no response from non-acclimated shoots (11,12). The 1-wk CA protocol was probably responsible for the good initial response of these *Humulus* accessions as well. We consider 40% the minimum acceptable recovery for storing accessions and most of the initial accessions tested exceeded that minimum. The 40% minimum was chosen based on our experience with the variation possible between tests. Longer cold acclimation (2 to 12 wks) is necessary for some genotypes of *Pyrus* and *Rubus* (4,5,6). Four accessions were tested with both the 1 and 2-wk CA tests and three had similar results with either CA regime but 'USDA 21110M' recovery increased from 9% with 1-wk CA to 46% with 2-wk CA. Increasing CA to 2 wks increased the mean *Humulus* shoot regrowth to 54 $\pm$ 13% and the range rose to a minimum of 37% and a high of 85%. All cultivars and wild accessions tested with 2-wks CA recovered at acceptable rates for storage (regrowth $\geq$ 40%) (Fig. 3). A recent study of cryopreservation of *Humulus* shoot tips found that slow cooling following 1 to 6 wks of a 12 °C/ 6 °C CA regime and DMSO-sucrose cryoprotectants produced no surviving shoot tips; a vitrification technique was also unsuccessful (18). In the same study the encapsulation-dehydration technique was very successful for the accessions tested (18). Our preliminary studies with encapsulation-dehydration were also successful and varied with genotype in the same recovery range as the slowly-cooled samples (data not shown). Our study shows that success of various cryopreservation techniques requires attention to critical

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points of the protocols. In the case of slow-cooling techniques, the type and length of CA and the cryoprotectant used are extremely important as well as the cooling rate. Deeper cold hardiness and increased regrowth in pear following cryopreservation are produced by alternating-temperature CA treatments that expose the plantlets to freezing temperatures (-1 °C) and shorter warm periods (22 °C) (5). Extended alternating-temperature CA periods of 2 or more weeks may also be needed for reaching optimum cold hardiness in some *Humulus* accessions. The efficacy of PGD as a cryoprotectant for use in slow-cooling protocols has been shown for cells and shoot tips of numerous genera (4,5,6,7,11,12,13,16). Storage of large germplasm collections require protocols that work well for the particular lab involved and that are successful for many types of plants. As germplasm curators prioritize the type and amount of germplasm to store, they also need to choose a technique that fits their facility. Personnel, equipment, expertise, plant type and available facilities influence which technique is most appropriate for a particular facility (14). When personnel are the rate limiting factor, slow cooling is a time, labor, and financially efficient method for cryopreserved storage of large numbers of accessions. Choosing a well-tested technique and applying it to a new genus can save development time and speed up storage of important plant collections. Over 30 *Humulus* accessions are now stored for base (long-term) germplasm preservation and the entire core collection (90+ accessions) will be stored in the near future.

Figure 2. Regrowth of *Humulus lupulus* shoot tips cryopreserved by slow cooling following 1-wk cold acclimation. Regrowth data were taken at 6 wks.

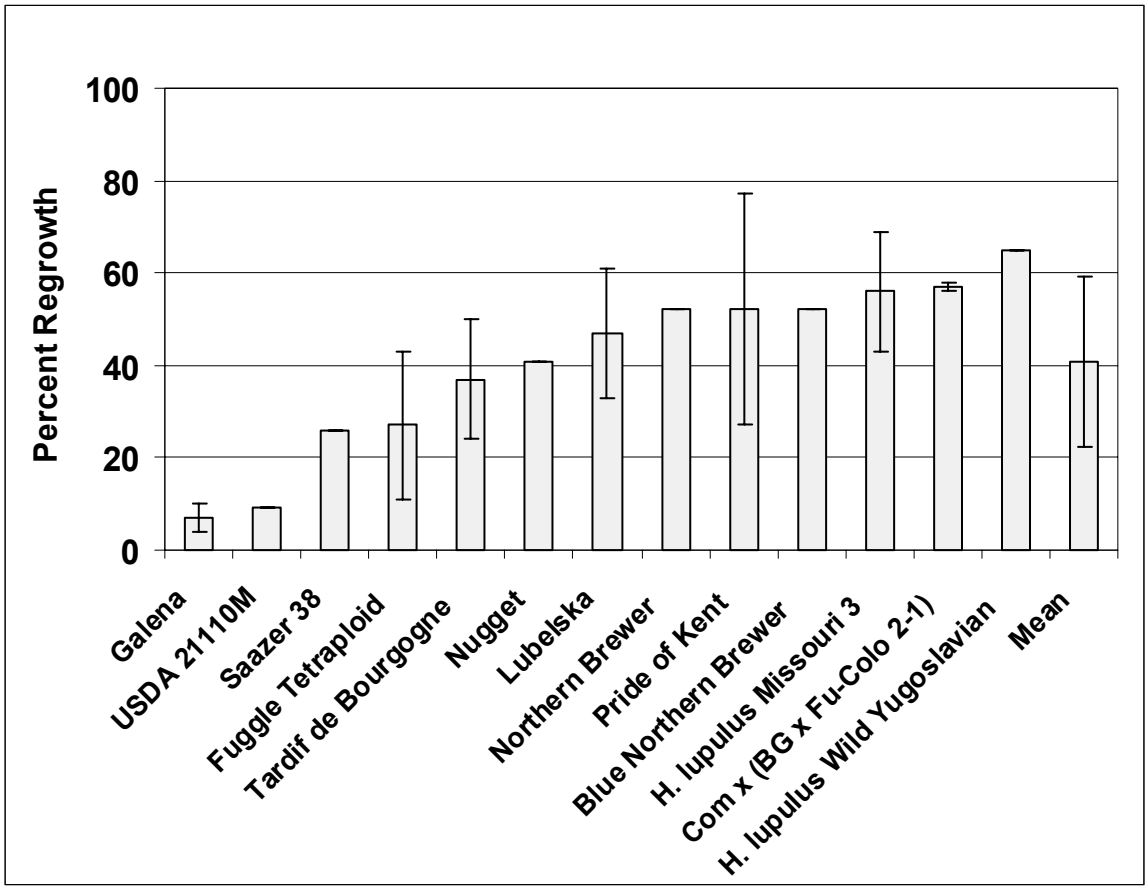

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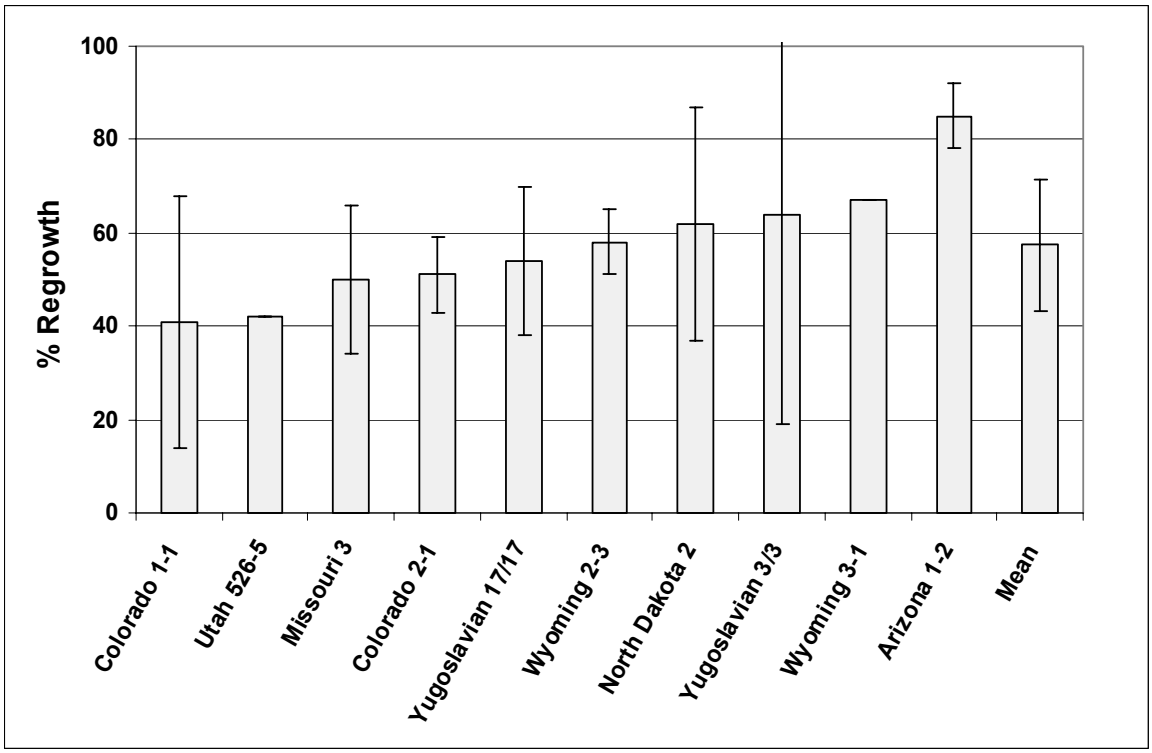
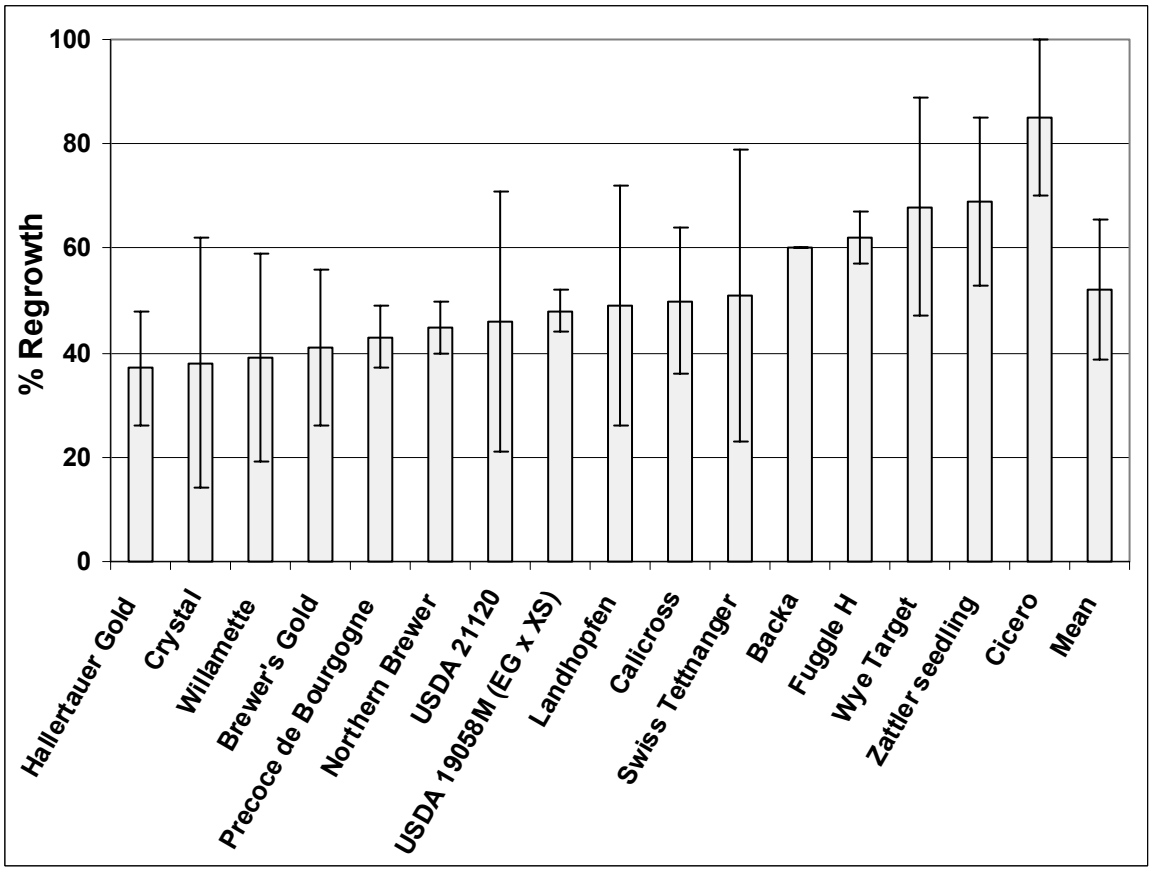
Figure 3. Ten wild *Humulus lupulus* accessions (upper graph) and 17 cultivars (lower graph) were tested 2 to 3 times (n=40 to 60) using 2-wks cold acclimation and cryopreserved by the standard slow cooling technique. Regrowth data were taken at 6 wks.





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